J. Physiol. (1962), **160**, *pp.* 221–233
With 5 text-figures
Printed in Great Britain

INFLUENCE OF NERVE-ENDING ACTIVITY AND OF DRUGS ON THE RATE OF PARALYSIS OF RAT DIAPHRAGM PREPARATIONS BY *CL. BOTULINUM* TYPE A TOXIN

BY R. HUGHES* AND B. C. WHALER†
From the Microbiological Research Establishment, Porton, near Salisbury, Wilts

(*Received* 3 *June* 1961)

Since the studies of Guyton & MacDonald (1947), Ambache (1948, 1949, 1951) and Burgen, Dickens & Zatman (1949) on the action of botulinum toxin it is generally agreed that the paralysis is caused by failure of release of the neurohumoral transmitter at the effector site (see also Brooks, 1954, 1956; Ambache & Lessin, 1955). So far, however, it has proved difficult to modify the course of the neuromuscular paralysis caused by the toxin. Guyton & MacDonald (1947), using intact animals, and Burgen *et al.* (1949), using the isolated rat diaphragm preparation, were unable to modify by drugs either the rate at which paralysis took place or the rate of recovery of the paralysed muscles. The only observations showing some modification of the rate of intoxication are those of Bronfenbrenner & Weiss (1924), who showed that the survival times of guinea-pigs given lethal doses of toxin could be considerably prolonged by anaesthesia. This suggested that inactivity delayed the onset of paralysis, as was borne out also by the following observations made by May & Whaler (1958) during experiments on the intestinal absorption of toxin. It was noticed that in intoxicated rabbits respiratory distress and inability to hold up the head often preceded paralysis of the limb muscles. Since the groups of muscles thus involved are in a state of continuous activity it seemed likely that the degree of activity might be important in determining the rate of onset of paralysis. This has now been confirmed on rat phrenic nerve–diaphragm preparations. Moreover, it has also been possible to alter appreciably the rate of onset of neuromuscular paralysis by means of drugs known to modify acetylcholine metabolism and function.

* Present address: Benger Laboratories Ltd., Holmes Chapel, Cheshire.
† Civil Service Senior Research Fellow; present address, Department of Physiology, Queen Elizabeth College, Campden Hill Road., London, W. 8.

METHODS

Clostridium botulinum toxin was prepared as described by May & Whaler (1958). Most batches of toxin had a toxicity of $0{\cdot}8$–$1{\cdot}0 \times 10^6$ LD_{50}/ml., when determined on 18–22 g male albino mice (Porton strain). For convenience 1 mouse-LD_{50} dose is here regarded as 1 unit of toxin and all toxin concentrations are given as units/ml.

Rat phrenic nerve-diaphragm preparations. These were obtained from 110 to 130 g rats, as originally described by Bülbring (1946) and were suspended in Krebs's bicarbonate-saline solution containing $0{\cdot}2$ % glucose and bubbled with 95 % O_2 and 5 % CO_2 (Krebs & Henseleit, 1932). Two organ baths, each containing 100 ml. of this saline solution at 35° C were used, one for a control and the other for a test preparation. The electrode system resembled that described by Garry & Wishart (1951), the phrenic nerve being threaded through a thin rubber diaphragm so that the preparation could be readily stimulated even when completely submerged. An 'Attree' double-channel stimulator was used both for direct (maximum 20 V, 2 msec) or indirect (maximum 10 V, 200 μsec) stimulation. The contractions of the muscle were recorded on a smoked drum by an isotonic lever.

When the muscle contractions had become of constant size, toxin was added to the bath. The bath concentration of toxin was usually 10,000 u./ml. and the rate of stimulation 12/min. Because of the danger of toxic aerosol entering the laboratory as a result of the vigorous oxygenation of the bath fluid, a suction pump maintained a continuous large air flow across the top of the organ bath and the effluent air was filtered before being discharged into the laboratory. Toxicity tests on mice showed that large losses of toxin from the medium did not occur during a 1–3 hr experimental period.

RESULTS

The normal course of paralysis. The time course of the intoxication process was as described by Burgen *et al.* (1949). With a bath concentration of 10,000 u./ml. there was first a latent period of 30–40 min during which no change occurred in the twitch tension. This was followed during the next 30–60 min by the decline and eventual disappearance of the response to indirect stimulation, although the muscle could still respond as usual to direct stimulation. Under these conditions there was little variation in 'paralysis time', i.e. the time from the addition of toxin to the disappearance of the response to indirect stimulation, and any such variation could be attributed to differences in toxin batches. For this reason in most cases the same batch of toxin was used throughout each particular series of experiments. For example, in one series of twenty preparations the mean paralysis time was 80 ± 11 min. Control preparations without added toxin contracted normally for at least 4 hr.

In five experiments highly purified toxin (at least 75 % pure; toxicity 190×10^6 LD_{50}/mg N) was used; 10,000 u./ml. of this also produced paralysis in 80–90 min. Thus the impurities present in our cruder toxin preparations did not appear to have influenced the course of the paralysis. This conclusion was supported by the finding that the dose–response curves for crude toxin culture (between 3,500 and 28,000 u./ml.) and for purified toxin (between 10,000 and 100,000 u./ml.) were very similar. Such

large changes in bath concentration of toxin produce relatively smaller changes in paralysis time; for example, with pure toxin, 10,000 u./ml. caused paralysis in 80 min whilst 100,000 u./ml. took 50 min.

When the muscle was stimulated at first directly for 10–90 min after adding toxin and then indirectly until paralysis occurred, the paralysis time remained unchanged at 70–90 min.

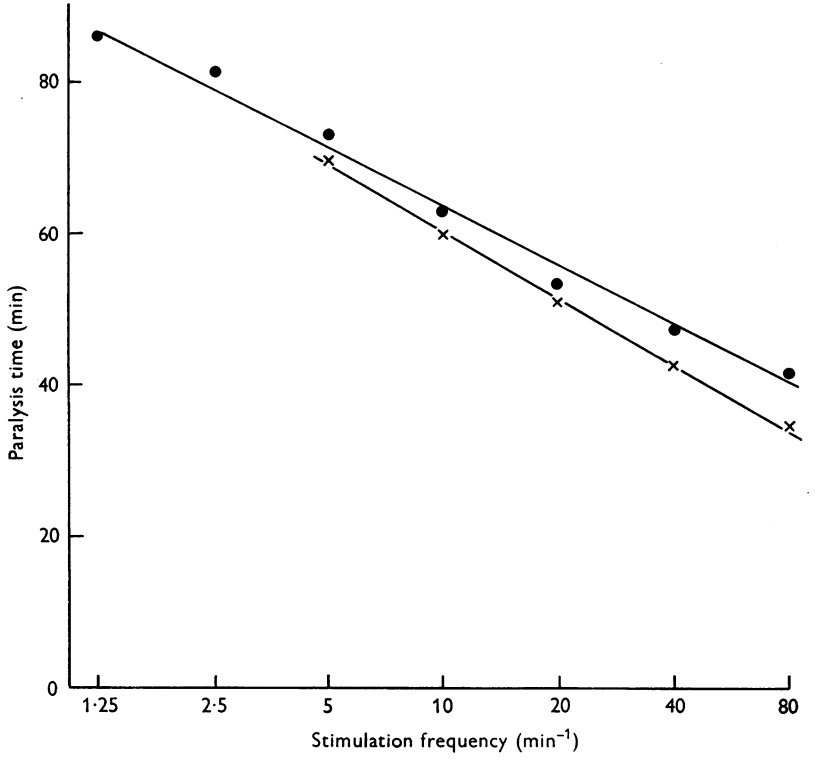

Fig. 1. Effect of frequency of indirect stimulation on the paralysis time. There is no appreciable increase in the rate of paralysis in preparations from rats preliminarily injected with toxin intravenously. ●—●, toxin to bath only; ×—×, toxin intravenously and subsequently in the bath. Semi-log. scale. For details see text.

The administration of toxin by the intravenous route would be expected to promote its delivery into the tissues and produce an effect much more rapidly than by diffusion through the muscle thickness from the bath fluid. It is clear from the following results that this is not so. Diaphragms were taken from rats given intravenous toxin (*ca.* 10,000 u./g body wt., corresponding to the usual bath concentration) 2–3 min before being killed. The excised diaphragms were then treated in the organ bath with the normal dose of toxin and became paralysed in approximately the normal

time; the mean for ten animals was 70 ± 15 min compared with the average 80 ± 11 min given above.

Effect of neuromuscular activity on the rate of paralysis. In 60 experiments when the rate of nerve stimulation was increased from 1 to 80/min the paralysis time was halved, as is shown in Fig. 1. A similar result was obtained in twenty preparations from rats which had received a preliminary injection of toxin intravenously just before setting up the

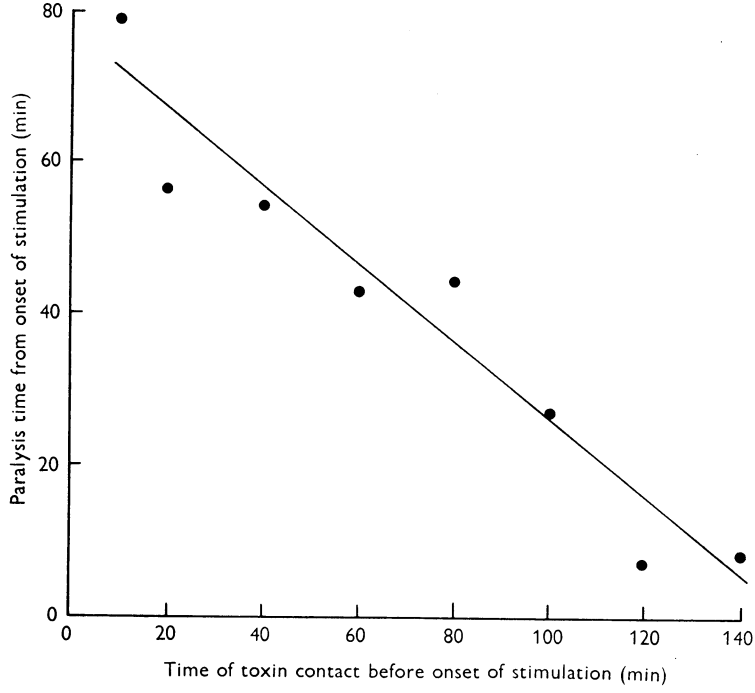

Fig. 2. The effect on the paralysis time of delaying the start of stimulation. Toxin was first added to the bath and, after the indicated interval, indirect stimulation was begun. The total time to paralysis is the sum of ordinate and abscissa at any one point.

diaphragm in the toxin solution. In control diaphragms there was no paralysis, but a fall in trace amplitude was noticed as the frequency of stimulation was increased.

In another series of 86 experiments the indirect stimulation (12/min) of the diaphragm was delayed for varying periods of time after the addition of toxin to the bath. The results of some typical experiments of this kind are illustrated in Fig. 2. With longer periods of inactivity there was a progressive increase in the total time taken for paralysis. In most cases mere contact of toxin with the diaphragm for 120 min was insufficient to

cause paralysis, but in seven out of eleven unstimulated diaphragms exposed to toxin for 160 min paralysis was complete when stimulation was begun. On a few occasions it was possible to have toxin in contact with the quiescent preparation for as long as 140–160 min, and when stimulation was restarted declining activity was observed for a further 10–40 min before paralysis was complete. As before, a previous intravenous dose of toxin did not appreciably hasten the rate of paralysis and the unstimulated preparations took twice as long to become paralysed (135 min against 70 min) as those stimulated continuously at 12/min.

Effect of varying the time of toxin contact. Burgen *et al.* (1949) showed that when toxin was removed from the organ bath only 5 min after its addition the time course of the paralysis remained unchanged. This implies a rapid entry and fixation of toxin.

In the present series of 48 experiments stimulation at 12/min was carried out during the period of toxin contact and after washing. The actual period of contact with the toxin varied between 2·5 and 80 min. Paralysis occurred in 174 ± 21 min (6 expts.) after only $2\frac{1}{2}$ min of toxin contact, and this paralysis time fell progressively to 88 min when contact was prolonged to 40 min. Longer contact periods produced little further reduction in paralysis time. In other experiments the diaphragms were left unstimulated during the period of toxin contact. Paralysis times were slightly longer than those found in stimulated preparations, but otherwise the results followed the same pattern.

Diaphragms from rats given a previous dose of toxin intravenously behaved very differently in this type of experiment. They were set up as before and toxin was added to the bath as usual. Removal of this toxin by replacing the bath fluid with fresh Krebs's solution after periods of 5–65 min resulted in only small changes in the paralysis times and these were close to the usual value of *ca.* 70 min found when the toxin was not washed out from the bath. Diaphragms stimulated at 12/min during a 5–60 min period of toxin contact became paralysed in about 75 min; those stimulated only after washing out the bath toxin (10–130 min after its addition) took longer to become paralysed and the results were essentially the same as those given earlier (Fig. 2) for experiments in which toxin was added only to the bath.

Effects of drugs

The experiments described above demonstrated quite clearly a relationship between neuromuscular activity and the rate of paralysis. From this it followed that the rate of paralysis by botulinum toxin might also be affected by pharmacologically induced changes in the physicochemical processes at the neuromuscular junction. Burgen *et al.* (1949) were unable

to alter the time course of paralysis by using drugs, but these attempts were made only when the paralysis was already far advanced. By contrast, we have studied the effect of drugs during the early stages of the intoxication, and have found that drugs can in fact hasten or delay the onset of paralysis.

Eserine. At a bath concentration of $3{\cdot}5 \times 10^{-6}$M eserine considerably increased the rate of paralysis. This enhancement of the effect of the toxin

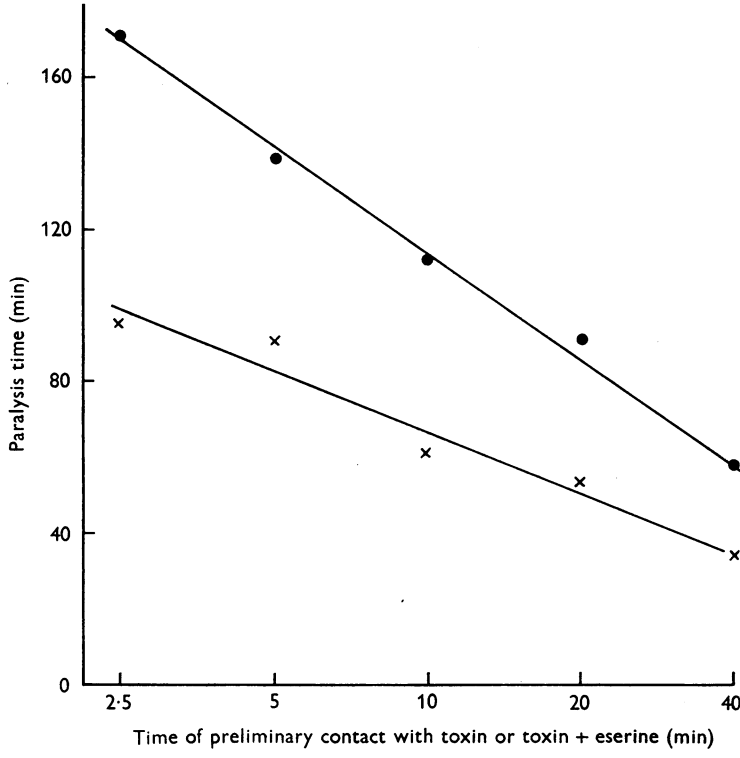

Fig. 3. Acceleration of botulinum paralysis by eserine. After a preliminary period of contact with toxin (and/or eserine) the bath fluid was replaced with fresh Krebs's solution. Indirect stimulation (12/min) was begun only after washing toxin (and/or eserine) from the bath. ●—●, toxin alone; ×—×, toxin with $3{\cdot}5 \times 10^{-6}$M eserine. Semi-log. scale.

was such as to reduce the paralysis time from 170 to *ca.* 100 min when toxin + eserine contact was maintained for only 2·5 min, after which the bath fluid was emptied and replaced with normal Krebs's saline solution. Figure 3 shows the results in experiments where the preparations were not stimulated during the period of toxin + eserine contact: similar differences between the two groups were obtained with preparations stimulated throughout, but the paralysis times were correspondingly shorter.

Likewise, preparations treated throughout with toxin–eserine mixture (i.e. without washing) and left unstimulated for varying periods were paralysed more rapidly than controls in which the eserine was omitted. The eventual paralysis in *ca.* 160–180 min of an unstimulated preparation has already been described; in the presence of eserine this time was approximately halved.

The effects of eserine were most marked in those experiments in which the toxin–eserine mixture was washed out shortly after being added (Fig. 3). When left for longer periods, with or without stimulation, the difference between the un-eserinized control and the test group was considerably reduced. In one series of experiments, for example, when washing was omitted, eserine reduced the time taken for complete paralysis from 80–85 min to only 60–70 min. This is a disproportionately small reduction but it suggests that eserine has its main effect in the early stages during which toxin becomes 'fixed' in some way to the tissue, and that it does not appreciably alter the actual intoxication processes by which the irreversible paralytic changes occur.

In these experiments the washing procedure consisted of emptying and re-filling the bath twice with Krebs's saline solution. Eserine is not easily removed from tissues and its presence after these washes could be demonstrated for some time by the persistence of Wedensky-type block.

Tetraethyl pyrophosphate (TEPP). In a few experiments TEPP (2×10^{-6} M) had an effect very similar to that of eserine.

Tubocurarine. At $2{\cdot}6 \times 10^{-6}$ M, administered before toxin, D-tubocurarine produced rapid paralysis to indirect stimulation. The toxin was then added, indirect stimulation was continued for varying periods of time and then the bath fluid was replaced with fresh Krebs's solution. The preparation recovered rapidly from the tubocurarine paralysis, and stimulation was continued until the subsequent toxin paralysis became complete. Tubocurarine by itself produced no striking change in the rate of development of the botulinum paralysis (Fig. 4).

A few experiments were carried out in which the curarized toxin-treated preparations were not stimulated until the toxin-tubocurarine mixture had been washed out. Indirect stimulation was then started and the experiment continued in the usual way. These quiescent preparations took longer to become paralysed than did those (mentioned above) in which stimulation of the curarized, and hence non-contracting, diaphragm was continuous. For example, with curarine + toxin contact periods of 5–20 min there was little difference between the two groups. When drug + toxin contact was maintained for 60 or 80 min the stimulated preparations were paralysed by the time the bath fluid was replaced with fresh Krebs's solution: those left unstimulated during this period, however, still required

a further 40–50 min before toxin paralysis was complete. It has already been mentioned that in the absence of tubocurarine stimulated preparations became paralysed more rapidly than unstimulated ones. Since this is also true of curarized preparations, the difference cannot be due to an effect of contraction of the muscle fibres, but suggests a more effective 'toxin fixation and block' at stimulated nerve-endings.

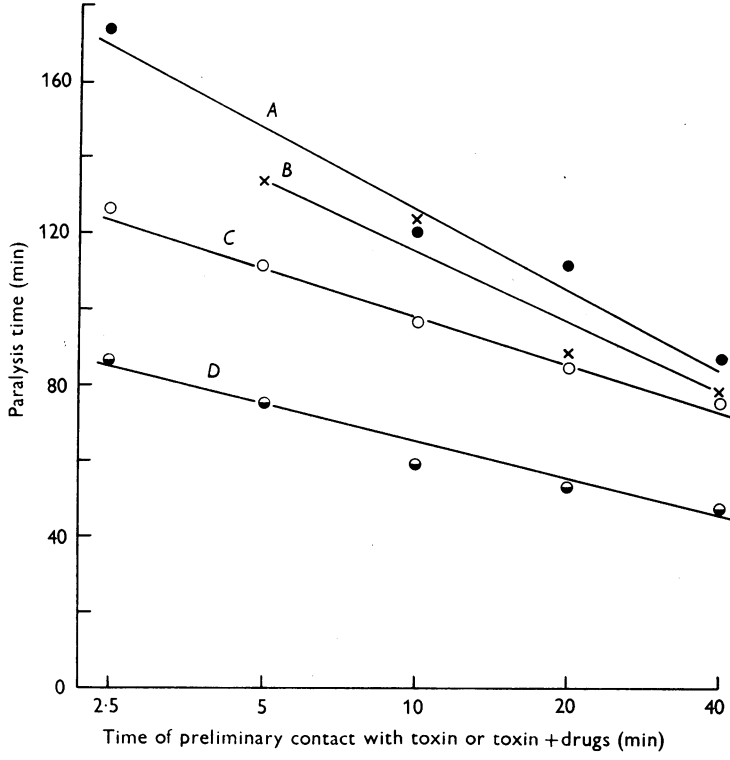

Fig. 4. Antagonism between the effects of D-tubocurarine and eserine on the paralysis time. The preparations were stimulated indirectly at 12/min throughout. *A*, ●—● toxin alone; *B*, ×—× toxin + D-tubocurarine ($2{\cdot}6 \times 10^{-6}$ M); *C*, ○—○ toxin + D-tubocurarine + eserine ($3{\cdot}5 \times 10^{-6}$ M); *D*, ◒—◒ toxin + eserine. Semi-log. scale.

Although tubocurarine did not appear to have any material effect on the development of intoxication it did substantially antagonize the hastening of paralysis produced by eserine (Fig. 4).

Decamethonium iodide. No modifying effect on the course of the toxin paralysis was observed with either paralysing or non-paralysing doses (range used 10–300 μg/ml.).

Atropine. Although not commonly regarded as having considerable blocking properties in voluntary muscle, atropine can produce neuro-

muscular paralysis if the dose is sufficiently large (*ca.* 10^{-3} M). In 48 experiments both paralysing and non-paralysing doses of atropine were used in conjunction with toxin. At a final concentration of $3{\cdot}5 \times 10^{-6}$ M, left in the bath with toxin until the end of the experiment, atropine slightly increased

TABLE 1. Effect of atropine concentration on the toxin paralysis time. Toxin was added after atropine and the time for complete paralysis to indirect stimulation (12/min) was recorded

Atropine concn. (M)	Mean paralysis time (min) (no. of expts. in brackets)	Increase over unatropinized controls (%)
Nil	80 ± 11 (20)	—
$3{\cdot}5 \times 10^{-6}$	93 (2)	16
$3{\cdot}5 \times 10^{-5}$	144 (4)	80
$3{\cdot}5 \times 10^{-4}$	157 (3)	96

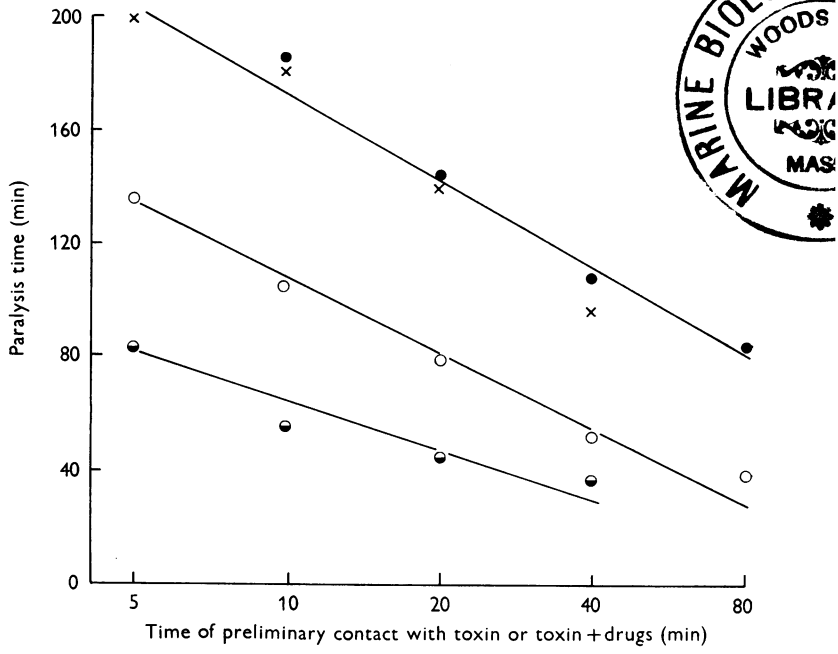

Fig. 5. The antagonism between atropine and eserine on the toxin paralysis time. Indirect stimulation (12/min) was started after the toxin (and/or drugs) had been washed from the bath. ○—○, toxin alone; ◒—◒ toxin + eserine ($3{\cdot}5 \times 10^{-6}$ M); ●—● toxin + atropine ($2{\cdot}4 \times 10^{-3}$ M); ×—× toxin + atropine + eserine. Semi-log. scale.

the time required for complete paralysis. This dose produced no demonstrable effect upon the twitch tension. Doses of $3{\cdot}5 \times 10^{-5}$ M or $3{\cdot}5 \times 10^{-4}$ M atropine considerably increased the paralysis time (Table 1).

In other experiments $3{\cdot}5 \times 10^{-3}$ M atropine was used. When the atropine

paralysis was complete, stimulation was stopped and toxin was added to the bath. The atropine-toxin mixture was left in contact with the unstimulated diaphragm for varying periods and indirect stimulation was resumed after washing out the mixture until the diaphragm was toxin-paralysed. After washing, recovery from the atropine block took 4–10 min. Again, atropine considerably prolonged the time for complete toxin paralysis (Fig. 5).

Atropine showed a marked ability to prevent the hastening of paralysis produced by eserine, restoring the paralysis time towards normal values (80–90 min) or even, at higher atropine concentrations, extending the time for paralysis beyond the normal. Figure 5 shows the marked protective effect of atropine on eserinized preparations. In other experiments less obvious interaction occurred, but even with 10^{-5} M atropine some degree of competition existed.

DISCUSSION

These experiments show quite clearly that the rate of botulinum intoxication can be modified *in vitro*.

First, there is a distinct relationship between stimulation of the motor nerve endings and paralysis of the diaphragm by toxin. Stimulation at 80/min brings about a more rapid paralysis than does 12/min; in the absence of stimulation paralysis is still further delayed. The experiments with diaphragms from rats previously given toxin intravenously (Fig. 1) show that this relationship cannot be due simply to muscular contraction causing an increased rate of entry of toxin into the muscles, but must be more closely dependent upon nerve-ending activity. It seems probable that intravenous toxin enters the muscle tissue spaces from blood vessels fairly rapidly, even after killing the animal, since replacing the bath fluid with fresh Krebs's solution after variable periods of toxin contact does not affect the paralysis time; in contrast, in the absence of previously injected toxin, the paralysis time is largely dependent upon the period of toxin contact.

The results with curarized diaphragms further implicate nerve activity in toxin fixation, as distinct from the muscular contractions themselves. Tubocurarine-paralysed preparations stimulated indirectly during contact with toxin + tubocurarine developed botulinic paralysis after the tubocurarine was washed out as readily as other stimulated preparations treated with the toxin alone (Fig. 4). In the former group there was neuromuscular paralysis and in the latter the muscle was contracting normally; in both cases there was nerve-ending activity and the toxin-paralysis times were similar. Furthermore, curarized preparations stimulated during drug-toxin contact became paralysed more rapidly than those not so

stimulated. Here in both groups there was neuromuscular paralysis and the differences between the two groups could only be due to presence or absence of nerve-ending activity.

These results would provide an explanation of the unpublished observations made in earlier experiments on rabbits (May & Whaler, 1958) and of those of Bronfenbrenner & Weiss (1924) on the effect of anaesthesia in prolonging survival in guinea-pigs injected with lethal doses of toxin.

Secondly, it has been shown that a number of drugs affect the response to toxin. Eserine and TEPP enhance the rate of paralysis; atropine delays it. Burgen *et al.* (1949) were unable to modify the rate of paralysis by drugs, but these were only added when some degree of paralysis was already obvious. If the drug is given at the same time as the toxin modification of the response is possible.

The exact mechanism of toxin action at cholinergic nerve endings is still uncertain. Guyton & MacDonald (1947), Ambache (1948, 1949, 1951; see Ambache & Lessin 1955) and Burgen *et al.* (1949) all attributed the intoxication to a failure of transmitter release at cholinergic nerve endings, leaving the effector cells (muscle or gland) fully responsive to their neuro-humoral transmitter and the nerve trunks proximal to the endings able to conduct normally. Burgen *et al.* (1949) showed that the choline-acetylating mechanism (of brain) was unaffected by toxin; they suggested that the transmission failure was perhaps due to a blockage of nerve impulses in the non-myelinated terminal portion of the motor fibres. Brooks (1954) supported this view and found that in excised guinea-pig diaphragms, paralysed to indirect stimulation, it was possible to release the expected normal amount of acetylcholine by strong direct stimulation of the muscle. As a result of this work Brooks emphasized the apparent normality of the acetylcholine stores, and in this connexion Thesleff (1960) has shown by electron photomicrography that the nerve-ending vesicles of intoxicated frog sartorius and cat tenuissimus muscles are of normal appearance.

In a later paper Brooks (1956) showed that spontaneous miniature end-plate potentials (e.p.p.s.) were abolished by the toxin. This finding led him to the view that the site of toxin action was in fact at the tips of the motor nerve endings, i.e. distal to the branchings of the terminal motor fibres suggested earlier by him and Burgen *et al.* as the point of attack, and the present results support this view.

We have also shown that factors which influence acetylcholine release or metabolism alter the rate of paralysis. For example, increased nerve-ending activity or anticholinesterase drugs accelerate the paralysis whilst atropine, which can antagonize the eserine effect (as does tubocurarine), delays the onset of paralysis.

These results suggest that the fixation of toxin, or its subsequent action, is enhanced, or perhaps even only made possible when the presynaptic region of the neuromuscular junction is at some stage in the normal depolarization–repolarization cycle. Inability to delay the paralysis beyond 140–180 min in these experiments by omitting to stimulate the nerve does not necessarily invalidate this view, because of the continuous junctional activity in the form of spontaneous miniature e.p.p.s (Fatt & Katz, 1952).

On this view, anticholinesterase drugs would serve to delay the repolarization phase after a stimulus and hence to maintain conditions favouring the action of the toxin for a longer period of time. The exact point at which this might occur is unknown, but seems most likely to be in the region of the membrane at the nerve ending through which the acetylcholine quanta are released. Evidence for the presence of so-called presynaptic acetylcholine receptors in these regions has been given by Masland & Wigton (1940) and it might be at these sites that acetylcholine promotes the action of the toxin. The effect of atropine could then be one of blocking these presynaptic receptors. Alternatively, atropine might act by reducing the release of acetylcholine, as suggested by Bülbring (1946). The antagonism of eserine by tubocurarine might also be due to a reduced acetylcholine output by tubocurarine (Naess, 1952) or by blocking of a presynaptic receptor site. However, we were unable to show that tubocurarine could, by itself, delay paralysis, and further information is required.

If one accepts the view that the membrane of the nerve endings plays a special part in the development of toxin paralysis, there are two obvious ways in which this might be possible. In one view, the physico-chemical changes at the synaptic region during the depolarization–repolarization cycle may determine the rate of entry of toxin molecules into the nerve endings. The toxin molecule itself may be able to take advantage of the increased permeability associated with depolarization. Being a large molecule, it might enter by the postulated gaps through which acetylcholine quanta are believed to be released. Having traversed this nerve membrane it could then play a further and perhaps specific role in blocking subsequent acetylcholine release and thus neuromuscular transmission.

In an alternative hypothesis the toxin molecules might become attached during the polarization cycle to the outside of the nerve ending in such a way as to block the gaps through which the quanta are liberated, eventually preventing any further release of acetylcholine. Here also changes in the amounts and timing of acetylcholine release and destruction would determine the toxin paralysis time. Only further work can decide between these or other hypotheses, but our present results show clearly the importance of acetylcholine metabolism in the action of botulinum toxin.

SUMMARY

1. Attempts have been made with the rat phrenic nerve–diaphragm preparation to alter the rate of its paralysis by *Cl. botulinum* toxin.

2. With frequencies of indirect stimulation up to 80/min, the time required for paralysis to develop is reduced. In the absence of stimulation paralysis develops more slowly but cannot be prevented.

3. The use of D-tubocurarine shows that the paralysis rate is independent of muscle-fibre activity and depends solely upon nerve-ending activity.

4. Anticholinesterase drugs accelerate the rate of paralysis and this action can be antagonized by atropine and by D-tubocurarine.

5. Atropine in high concentration can increase the time required for paralysis to occur.

6. The results suggest that the rate of toxin paralysis is primarily dependent upon acetylcholine metabolism at nerve endings.

An account of this work has been submitted (by R.H.) to the University of Wales in part fulfilment of the requirements for a Master's degree.

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